BBA 65701

MECHANISM OF CARDIAC GLYCOSIDE INHIBITION OF THE (Na^+-K^+)-DEPENDENT ATPase FROM CARDIAC TISSUE

HIDEO MATSUI AND ARNOLD SCHWARTZ*

Department of Pharmacology, Baylor University College of Medicine, Houston, Texas 77025 (U.S.A.)

(Received August 21st, 1967)
(Revised manuscript received November 9th, 1967)

SUMMARY

Specific digoxin binding to a (Na^+-K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) from cardiac tissue was determined by use of [^{3}H]digoxin. The binding required ATP and Mg^{2+}, was stimulated by Na^+ and depressed by K^+. Active cardiac glycosides significantly diluted the [^{3}H]digoxin binding, while inactive ones had no effect. The binding was also observed in the presence of acetyl phosphate substituted for ATP; in this case Na^+ had no stimulatory effect while K^+ still depressed the binding. Considering the similarity of the increase and decrease of both the digoxin binding and the phosphorylated intermediate of the ATPase under various conditions, these results suggest that digoxin binds with the phosphorylated conformation of the enzyme. The mechanism of digoxin inhibition of the ATPase is explained by the stability of the digoxin–intermediate complex.

INTRODUCTION

The (Na^+-K^+)-dependent ATPase (ATP phosphohydrolase, EC 3.6.1.3) associated with active cation transport, is inhibited by cardiac glycosides[1–4]. The specificity of inhibition of the (Na^+-K^+)-ATPase by active cardiac glycosides, is not mimicked by any other known inhibitor. One of the unique characteristics of the cardiac glycoside-induced inhibition is the antogonism between the glycoside and K^+, *i.e.*, a decrease of the inhibition results from an elevation of the potassium level[5,6]. The antagonism is, however, not due to simple competition between the cardiac glycoside and K^+ for a receptor site. Moreover, elevation of Na^+ effects an increase in glycoside inhibition of the (Na^+-K^+)-ATPase. This phenomenon suggests the participation of Na^+ in the digitalis–K^+ interaction. In a previous report[7], dependency of the K_i for cardiac glycoside-induced inhibition on the Na^+/K^+ ratio, was demonstrated kinetically.

In order to examine this complex relationship in depth, a study of [^{3}H]digoxin binding to a heart (Na^+-K^+)-ATPase was undertaken. In a preliminary communi-

* Please address all communications to Dr. Schwartz.

cation[8], we reported that the binding of [^{3}H]digoxin is ATP dependent, is increased by Na^+ and decreased by K^+. The present communication describes the specificity of digoxin binding with particular reference to dependence either on ATP or on acetyl phosphate. The latter suggests that digoxin probably interacts with the phosphorylated state of the (Na^+-K^+)-ATPase.

MATERIALS AND METHODS

Preparation and assay of (Na^+-K^+)-ATPase

The preparation and assay of a (Na^+-K^+)-dependent ATPase from calf heart have been previously described[9]. More than 95% of the total ATPase activity was (Na^+-K^+)-dependent and cardiac glycoside-sensitive. The specific activities of the (Na^+-K^+)-ATPase of the preparations employed varied from 13 to 29 μmoles P_i per mg protein per h at 37°.

Determination of [^{3}H]digoxin binding to (Na^+-K^+)-ATPase

The standard reaction mixture contained in a polycarbonate centrifuge tube (16 mm × 76 mm), consisted of 1 mg (approx.) of enzyme protein, 2 mM ATP (Tris salt), 5 mM $MgCl_2$, 1 mM EDTA–Tris, 100 mM NaCl, 50 mM Tris–HCl (pH 7.4) and 10^{-7} M [^{3}H]digoxin (specific activity: 140 μC/μmole) in a total volume of 2 ml. The reaction was started by the addition of the enzyme and was incubated for 3 min at 37°. The tube was quickly transferred to a pre-warmed rotor (Spinco No. 40) and centrifuged for 3 min at 105 000 × *g*. The supernate was discarded and the droplets attached to the tube wall were removed with a Kimwipe. The pellet was dissolved in 0.3 ml of 0.2 M NaOH in a water bath at 100°. The entire solution was transferred to a counting vial containing 15 ml of scintillation medium (100 g naphthalene, 6 g of 2,5-diphenyloxazole in a total of 1 l of dioxane), and the radioactivity was counted in a Beckman liquid scintillation spectrometer. The binding of [^{3}H]digoxin was expressed as pmoles digoxin per mg protein.

Chemicals and reagents

[^{3}H]Digoxin was kindly supplied by Burroughs-Wellcome and Co., Tuckahoe, N.Y. Ouabain was obtained from Mann Research Laboratories, Inc., New York, N.Y. Scillaren A and hexahydroscillaren A were gifts from Sandoz Pharmaceuticals, Hanover, N.J. Prednisolone–sodium succinate was purchased from Sigma Chemical Co. Acetyl phosphate (dilithium salt) and carbamyl phosphate (dilithium salt) were obtained from California Biochemical Co., Los Angeles, Calif., and were converted to the respective Tris salts by passing through a Dowex 50 (H^+) column and neutralizing with Tris. Other chemical reagents were obtained or prepared as described previously[9].

RESULTS

Specificity of [^{3}H]digoxin binding to the (Na^+-K^+)-ATPase

Since the final concentration of [^{3}H]digoxin used in the experiments was very low, the recovery of radioactivity was first determined in order to ascertain the precision of the procedure. The results of Fig. 1 indicate a consistent complete recovery of counts.

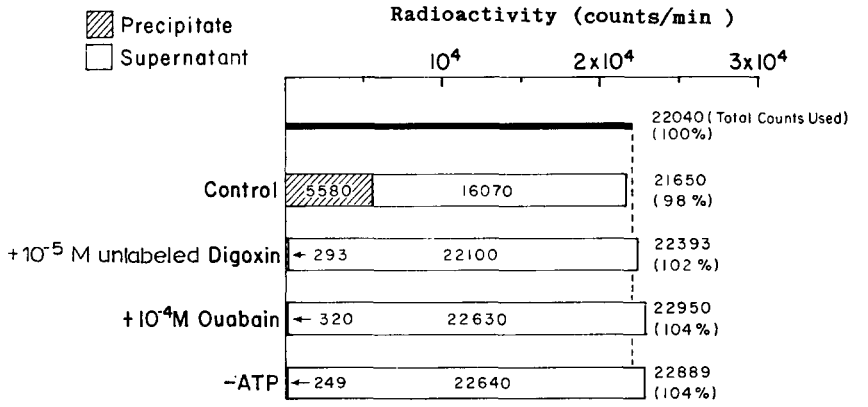

Fig. 1. Specificity of [^{3}H]digoxin binding to (Na+-K+)-ATPase and recovery of radioactivity. The standard assay conditions given in METHODS were used with 10^{-7} M [^{3}H]digoxin (specific radioactivity: 140 μC/μmole) and 1 mg protein of the enzyme (specific activity: 29.3 μmoles P_i per mg per h). Radioactivity in the supernate was also counted. Counts in precipitate and supernate were compared, after correction of quenching differences between precipitate and supernate.

Employing the standard experimental conditions, the radioactivity of [^{3}H]-digoxin found in the precipitate was more than one-fourth of the total radioactivity (Fig. 1). The precipitate-bound radioactivity, however, was diluted to a very low level by the addition of excess cold digoxin or ouabain. Omission of ATP also decreased the bound radioactivity to a similar low level. These results suggest that the binding of [^{3}H]digoxin to a (Na+-K+)-ATPase preparation is a specific and active one, and not due to simple adsorption. The decrease by ouabain of [^{3}H]digoxin binding is explained by assuming that the same binding site exists for digoxin and ouabain.

The diluted level of digoxin binding by ouabain was always the same as the level

TABLE I

COMPARISON OF SPECIFIC AND NON-SPECIFIC BINDING OF [^{3}H]DIGOXIN TO (Na+-K+)-ATPase, AT DIFFERENT DIGOXIN CONCENTRATIONS

Conditions were the same as in Fig. 1. The values in parentheses refer to the digoxin binding calculated as pmoles digoxin per mg protein, using the specific activity of [^{3}H]digoxin of 113 counts/min per pmole.

Concn. of [^{3}H]digoxin (M)	*[^{3}H]Digoxin binding (counts/min per mg protein)*		
	(A) Total binding	*(B) Non-specific binding (+10^{-4} M ouabain)*	*(A − B) Specific binding*
10^{-8}	910	27	881 (7.8)
$3 \cdot 10^{-8}$	2380	92	2288 (20.3)
10^{-7}	5000	295	4705 (41.6)
$3 \cdot 10^{-7}$	6616	798	5818 (51.5)
10^{-6}	8039	2378	5661 (50.1)

TABLE II

DEPENDENCY OF [^{3}H]DIGOXIN BINDING UPON ATP AND Mg^{2+}

Conditions were the same as in Fig. 1, except for specific activity of the (Na^+-K^+)-ATPase, 22.8 μmoles P_i per mg protein per h.

Omission	*Digoxin binding (pmoles digoxin per mg protein)*	
	— Ouabain	*+Ouabain, 10^{-4} M*
Complete system	41.5	2.4
— ATP	2.3	2.1
— Mg^{2+}	6.1	2.1
— ATP, — Mg^{2+}	2.2	1.9

obtained by the omission of ATP. Therefore, this low level is considered to represent non-specific binding of digoxin due probably to an adsorption of water containing [^{3}H]digoxin to the precipitate. This aspect is clearly demonstrated in Table I. The non-specific binding, *i.e.*, the binding in the presence of excess ouabain, was proportionately increased with increasing digoxin concentration in the reaction mixture. In other words, the percent of non-specific binding to the total digoxin used was at a constant level (around 1.3%) throughout the concentration changes of digoxin from 10^{-8} M to 10^{-6} M. On the other hand, specific binding, *i.e.*, the difference between total and non-specific binding, reached a saturation level at concentrations more than 10^{-7} M digoxin. The molar equilibrium constant of the specific binding was $3 \cdot 10^{-8}$, obtained by the method of KILROE-SMITH[10] (a modification of the Dixon method). The maximum

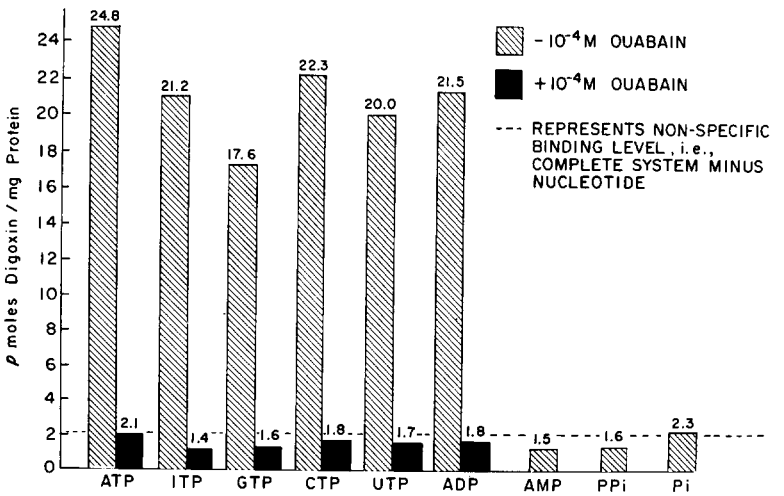

Fig. 2. Nucleotide specificity of [^{3}H]digoxin binding. All nucleotides except for ATP, PP_i and P_i were sodium salts, and concentration of each was 2 mM. Specific activity of the (Na^+-K^+)-ATPase used was 12.7 μmoles P_i per mg protein per h. Other conditions were the same as given in Fig. 1.

TABLE III

EFFECT OF Na^+ AND K^+ ON [^{3}H]DIGOXIN BINDING

Conditions were the same as in Fig. 1, except for the addition or omission of Na^+ and K^+ given in the table and specific activity of the (Na^+-K^+)-ATPase, 22.8 μmoles P_i per mg protein per h. The values in parentheses refer to experiments carried out in the presence of 10^{-4} M ouabain.

System		*Digoxin binding (pmoles digoxin per mg protein)*	
100 mM Na^+	*10 mM K^+*	*+ATP*	*−ATP*
−	−	21.6	4.7 (1.9)
+	−	42.6	2.2
+	+	9.1	2.2
−	+	8.1 (2.2)	1.8

level of binding was 55 pmoles/mg of protein. This level, however, was dependent on the (Na^+-K^+)-ATPase activity of the preparation. Mitochondrial ATPase from heart muscle did not show significant binding of digoxin.

Effect of Na^+ and K^+ on the ATP-dependent digoxin binding

The specific binding of digoxin required not only ATP, but also Mg^{2+} (Table II). Other nucleoside triphosphates or ADP were also effective for the digoxin binding (Fig. 2). AMP or PP_i were ineffective. In the presence of Na^+, P_i appears to be only slightly effective. As for divalent cations other than Mg^{2+}, like the (Na^+-K^+)-dependent ATP hydrolysis, Mn^{2+} was effective in supporting the binding of [^{3}H]digoxin.

The effect of Na^+ and K^+ on the ATP-dependent digoxin binding is shown in Table III. Although Na^+ was not an absolute requirement for the binding, it significantly increased the binding. On the other hand, K^+ decreased the binding. The digoxin binding therefore appeared to be dependent on both Na^+ and K^+ levels. The pattern of increase or decrease of the digoxin binding under the various ionic conditions described is similar to that of the formation of the phosphorylated intermediate of the

TABLE IV

EFFECT OF Na^+ AND K^+ ON [^{3}H]DIGOXIN BINDING: COMPARISON OF ATP AND ACETYL PHOSPHATE AS SUBSTRATE

Acetyl phosphate was used in place of ATP in the presence or absence of Na^+ and K^+. Specific activity of the enzyme was 22.3 μmoles P_i per mg protein per h. Other conditions were the same as in Fig. 1. The values in parentheses refer to experiments carried out in the presence of 10^{-4} M ouabain.

System		*Digoxin binding (pmoles digoxin per mg protein)*	
100 mM Na^+	*10 mM K^+*	*2 mM ATP*	*2 mM Acetyl-phosphate*
−	−	14.7	34.9
+	−	38.1 (2.4)	28.4 (2.4)
−	+	6.2	3.9

$(Na^+\text{-}K^+)$-ATPase[11–13]. Consequently, these results indirectly suggest that digoxin binds only with a phosphorylated conformation of the $(Na^+\text{-}K^+)$-ATPase.

Digoxin binding in the presence of acetyl phosphate

It has been reported that a K^+-dependent phosphatase activity associated with the $(Na^+\text{-}K^+)$-dependent ATPase, is ouabain-sensitive[14–18]. The same phosphorylated intermediate of the $(Na^+\text{-}K^+)$-ATPase formed from either ATP or acetyl phosphate was also recently suggested[19]. The digoxin binding was, therefore, compared for ATP and acetyl phosphate dependency. As shown in Table IV, digoxin did bind to the enzyme in the presence of acetyl phosphate in place of ATP. In contrast to the ATP-dependent binding, the acetyl phosphate-dependent binding of digoxin was very high even in the absence of Na^+, and in fact Na^+ slightly decreased the binding. K^+ caused a marked depression of the acetyl phosphate-dependent binding similar to the binding in the presence of ATP. Carbamyl phosphate, another known substrate for the K^+-dependent phosphatase[16,20], also supported digoxin binding similar to that observed in the presence of acetyl phosphate.

Dilution of [^3H]digoxin binding by active cardiac glycosides

Since excess ouabain diluted the specific digoxin binding to the enzyme, the possible dilution effects of other cardiac glycosides and other non-cardiotonic steroids were tested at various concentrations. The active cardiac glycosides ouabain and

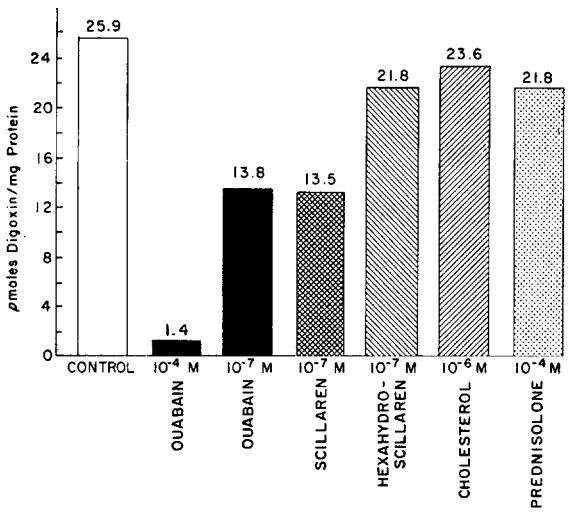

Fig. 3. Dilution of [^{3}H]digoxin binding by cardiac glycosides and steroids. Cardiac glycoside or non-cardiac steroid was added in the reaction mixture containing [^{3}H]digoxin in the concentrations shown in the figure. Specific activity of the enzyme was 12.7 μmoles P_i per mg protein per h. Other conditions were the same as in Fig. 1.

scillaren A (K_i values for $(Na^+\text{-}K^+)$-ATPase inhibition are of the same order as digoxin), decreased the binding to half of the original level at a concentration of 10^{-7} M. Hexahydroscillaren A, which is a less effective inhibitor for the ATPase[5], showed only minimal dilution of the [^{3}H]digoxin binding (Fig. 3). Non-cardiotonic steroids, such

as cholesterol and prednisolone, had no effect on the digoxin binding even at high concentrations.

DISCUSSION

The binding of digoxin to the (Na^+-K^+)-ATPase appears to require the presence of ATP and Mg^{2+} in the reaction system, which suggests that digoxin cannot bind to the free enzyme. Before they become reactive, either the enzyme or digoxin must be modified in some manner by ATP and Mg^{2+}. The following represent some possible modifications: (1) Digoxin (Dig) binds only after the formation of a phosphorylated intermediate of the enzyme (E-P) by ATP: Dig + E-P $\rightleftharpoons$ Dig·E-P. (2) Digoxin is phosphorylated by ATP; the phosphorylated digoxin (Dig-P) binds to the enzyme: Dig-P + $E \rightleftharpoons$ Dig-P·E. (3) The binding of ATP to the enzyme at a site other than the active center, changes the conformation of the enzyme to become accessible to digoxin: Dig + E·ATP $\rightleftharpoons$ Dig·E·ATP. The second possibility is analogous to the first. However, there is no evidence to support the presence of a phosphorylated digoxin or any other cardiac glycoside. The third possibility is in agreement with the data describing the dependency on ATP and Mg^{2+}. However, this offers no explanation for the specific effect of Na^+ and K^+ on the ATP-dependent binding nor to the binding which occurs in the presence of acetyl phosphate. Consequently, the first possibility fits best with both the present data and with the current concept concerning the phosphorylated intermediate of the (Na^+-K^+)-ATPase[11–13]. Evidence for the presence of a phosphorylated intermediate possessing an acyl phosphate linkage has in fact been reported[21,22]. Assuming that digoxin binds to this intermediate, the effect of Na^+ and K^+ on the binding level of digoxin can be explained by the known effect of Na^+ and K^+ on the level of the intermediate. The increase of the digoxin binding by Na^+ is due to acceleration by Na^+ of the formation of the intermediate from the enzyme and ATP, while the decrease of the binding by K^+ is due to the depression by K^+ of the intermediate level, *via* the activation of dephosphorylation of the intermediate. A K^+-dependent acyl phosphatase activity is thought to be a part of the (Na^+-K^+)-ATPase[14–18]; the formation of the same phosphorylated intermediate from acetyl phosphate as that derived from ATP was recently reported[19]. Therefore, digoxin binding in the presence of acetyl phosphate, as in the presence of ATP, is explained by the same mechanism. This is further exemplified by the fact that Na^+ does not increase the acetyl phosphate-dependent binding, while K^+ decreases the binding.

The apparent discrepancy of nucleotide specificities between the (Na^+-K^+)-dependent hydrolysis[9] and the digoxin binding may be explained in at least two ways. Firstly, the assay of hydrolysis activity is equivalent to the determination of velocity of the phosphorylation by a nucleotide, which is assumed to be the rate-limiting step. Hydrolysis of the nucleotide (*i.e.*, in the presence of Na^+ and K^+) would be undetectably small if the velocity of phosphorylation by the particular nucleotide were far less than that by ATP. In the case of the digoxin binding reaction system, however, even though the phosphorylation velocity of the specified nucleotide were much lower than that of ATP, the intermediate would still accumulate to some extent, since the system does not contain K^+. Phosphorylation of the enzyme would, therefore, be faster than any possible spontaneous hydrolysis of the intermediate and digoxin binding could

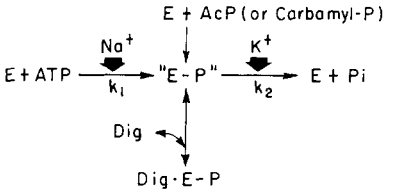

Fig. 4. Inhibition mechanism of a (Na^+-K^+)-ATPase by cardiac glycoside. E, enzyme; E-P, phosphorylated intermediate of the enzyme; Dig, digoxin; Dig·E-P, digoxin–phosphorylated enzyme complex; k_1 and k_2 represent velocity constants of Na^+-accelerated phosphorylation step and K^+-accelerated dephosphorylation step, respectively.

readily be observed. Furthermore, digoxin, in combining in a tight manner with even relatively few molecules of the intermediate, would tend to shift the reaction equilibrium to the right (Fig. 4) increasing the formation of intermediate, thus allowing for measurable binding. The second possible explanation is that the various nucelotides support digoxin binding by effecting a conformational change in the protein allowing for a favorable drug–enzyme interaction. This, however, would not preclude the formation of a phosphorylated intermediate.

Simultaneous stoichiometric determination of the amounts of the phosphorylated intermediate and the bound digoxin has not as yet been carried out. The present data, however, indicate that about 2 pmoles of [^{3}H]digoxin are bound per unit of enzyme (μmoles P_i per mg protein per h at 37°). This compares with the previously reported 2 pmoles of ^{32}P intermediate per unit of enzyme[9,12] and suggests a 1:1 relationship.

The relationships concerning the digoxin binding and inhibition of ATPase are represented in Fig. 4. Formation of E-P, either in the presence or absence of Na^+, and hydrolysis of E-P in the presence of K^+, represent the (Na^+-K^+)-dependent ATPase system. Digoxin presumably binds to E-P, forming a complex. The binding site for the glycoside appears to be neither the binding site for Na^+ nor for K^+. The Dig·E-P complex must be relatively stable in order to explain the glycoside-induced inhibition of the (Na^+-K^+)-ATPase. It would appear therefore that the cardiac glycoside is an allosteric inhibitor effecting a stabilization of some intermediary form of the enzyme. This is consistent with data reported recently by POST AND SEN[23].

NOTE ADDED IN PROOF (Received December 27th, 1967)

Recent experiments indicated that active cardiac glycosides, under certain conditions, can significantly bind to the dephospho-form of the enzyme in the absence of Na^+. The latter in fact inhibits the binding. These data fear on the allosteric nature of the (Na^+-K^+)-ATPase (manuscript in preparation).

ACKNOWLEDGEMENTS

Supported by grants from the National Institutes of Health (HE-07906-05 and HE-05435-07), the National Science Foundation (GB-4070) and the Texas and Houston Heart Associations.

A.S. is Career Development Awardee of the National Institutes of Health (K3HE 11 875-03).

REFERENCES

1 J. C. SKOU, *Physiol. Rev.*, 45 (1965) 596.
2 H. J. SCHATZMANN, *Helv. Physiol. Acta*, 11 (1953) 346.
3 R. L. POST, C. R. MERRITT, C. R. KINSOLVING AND C. D. ALBRIGHT, *J. Biol. Chem.*, 235 (1960) 1796.
4 J. C. SKOU, *Biochim. Biophys. Acta*, 42 (1960) 6.
5 E. T. DUNHAM AND I. M. GLYNN, *J. Physiol.*, 156 (1961) 274.
6 R. L. POST AND C. D. ALBRIGHT, in A. KLEINZELLER AND A. KOTYK, *Membrane Transport and Metabolism*, Academic Press, New York, 1961, p. 219.
7 H. MATSUI AND A. SCHWARTZ, *Biochem. Biophys. Res. Commun.*, 25 (1966) 147.
8 H. MATSUI AND A. SCHWARTZ, *Federation Proc.*, 26 (1967) 398.
9 H. MATSUI AND A. SCHWARTZ, *Biochim. Biophys. Acta*, 128 (1966) 380.
10 T. A. KILROE-SMITH, *Biochem. J.*, 100 (1966) 334.
11 R. W. ALBERS, S. FAHN AND G. J. KOVAL, *Proc. Natl. Acad. Sci. U.S.*, 50 (1963) 474.
12 R. L. POST, A. K. SEN AND A. S. ROSENTHAL, *J. Biol. Chem.*, 240 (1965) 1437.
13 R. GIBBS, P. M. RODDY AND E. TITUS, *J. Biol. Chem.*, 240 (1965) 2181.
14 J. D. JUDAH, K. AHMED AND A. E. M. MCLEAN, *Biochim. Biophys. Acta*, 65 (1962) 472.
15 M. FUJITA, T. NAKAO, Y. TASHIMA, N. MIZUNO, K. NAGANO AND M. NAKAO, *Biochim. Biophys, Acta*, 117 (1966) 42.
16 H. BADER AND A. K. SEN, *Biochim. Biophys. Acta*, 118 (1966) 116.
17 K. NAGAI, F. IZUMI AND H. YOSHIDA, *J. Biochem.*, 59 (1966) 295.
18 R. W. ALBERS AND G. J. KOVAL, *J. Biol. Chem.*, 241 (1966) 1896.
19 C. H. BOND, H. BADER AND R. L. POST, *Federation Proc.*, 25 (1966) 567.
20 H. YOSHIDA, F. IZUMI AND K. NAGAI, *Biochim. Biophys. Acta*, 120 (1966) 183.
21 K. NAGANO, T. KANAZAWA, N. MIZUNO, Y. TASHIMA, T. NAKAO AND M. NAKAO, *Biochem. Biophys. Res. Commun.*, 19 (1965) 759.
22 L. E. HOKIN, P. S. SASTRY, P. R. GALSWORTHY AND A. YODA, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 177.
23 R. L. POST AND A. K. SEN, *Federation Proc.*, 26 (1967) 592.